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In vitro propagation of the gum arabic tree (*Acacia senegal* (L.) Willd.) 1. Developing a rapid method for producing plants

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Abstract. The method described herein permitted production of three to four micropropagules of *Acacia senegal* from one uninodal explant. The explants were taken from plants produced either in a sterile environment or during four years in a greenhouse. Zeatin or 6-benzylaminopurine (BAP) were mixed, at different concentrations, with Murashige and Skoog's medium (MS) of which the amount of macroelements was divided in half (MS mod.). At a concentration of 5.0×10^{-5} M, zeatin produced a better multiplication rate after 60 d for the two types of plant stock than did BAP. A two stage process was necessary to obtain a rooting rate of the small cuttings close to 100%. The first stage, called induction, consisted of leaving the cuttings for 6 to 12 d on a Jordan's medium (JN) of which the amount of macroelements was reduced by half (JN mod.) and in which NAA at a concentration of 5.0×10^{-5} M was added. The second stage, called root extension, required that the small cuttings be planted on this second hormone-free medium. Roots appeared after a few days. Acclimatization in a greenhouse occured with a survival rate of close to 100% when the rooted *in vitro* plants were transplanted to pots containing a mixture of vermiculite and top soil (1:1; v/v).

Abbreviations : BAP, 6-benzylaminopurine; NAA, a-Naphthaleneacetic acid; JN, Jordan *et al.* (1978) medium; MS, Murashige and Skoog (1962) medium; mod., modified.

Introduction

Acacia senegal (L.) Willd. is an economically and ecologically important species. It produces gum arabic, which is highly sought for its diverse qualities by the food industry and others (C.C.I., 1978). The tree improves soil mineral content through symbiosis with *Rhizobium* and mycorrhiza (Colonna *et al.*, 1990) and is among the local woody species most often used for reforestation in semi-arid zones.

Spread over a vast geographic area (Giffard, 1966), *Acacia senegal* demonstrates great intraspecific variability with respect to gum production. Yield per tree can vary from 0 to 1 kg per year (Sène, 1989) with an average of at least 200 g per year for a productive stand (Dione, 1989).

In order to improve agrosylvicultural systems which use this species as a principal arboreal component, it is necessary to make available high yielding individuals. The selection of individuals and the refining of plant multiplication techniques is a way which can permit attainment of this objective. The works of Badji *et al.* (1991) and Danthu *et al.* (1992a) demonstrated the feasibility of producing horticultural cuttings of *Acacia senegal*, as well as its limits: the most responsive planting stock is taken from cuttings 15 cm long (with 12 to 15 nodes) without leaves and a diameter of 10±6 mm (wood of 2 to 4 years). The development of the root system is better when the cutting is taken during the rainy season (June-October) than during the dry season.

Micropropagation, which is often used successfully for the multiplication of herbaceous and woody plants, represents an interesting alternative for this species. However, among woody plants, regeneration from adult subjects is often not successful directly using this method (Bonga, 1982) and requires a rejuvenation of the plant material (Bonga, 1987; Franclet *et al.*, 1987). Vegetative multiplication of woody species has, however, been obtained from mature trees of such species as *Sequoia sempervirens* (Boulay, 1980), *Tectona grandis* (Gupta *et al.*, 1980) and *Acacia albida* (Gassama and Duhoux, 1986) as well as others.

In general, refining a cloning method from explants emanating from seedlings or subjects maintained in a juvenile state is a prerequisite for producing microcuttings from adult trees. This is the objective of the work herein reported as applied to the gum arabic tree.

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Materials and methods

Planting Stock.

Two different kinds of planting stock were used.

(i) Juvenile stock from seedlings produced under sterile conditions. The seeds originating from northern Senegal were harvested from selected trees. The seeds were stored in a cold storage room (5±1° C, 55±5% RH) and treated with an insecticide (Hexapoudre 25, containing 6% hexachlorocyclo- hexane) until use. They were soaked for 14 min in 95% H_2SO_4 to render uniform the development of the seeds as well as to disinfect them (Vogt and Palma, 1991; Danthu *et al.*, 1992b). After a series of rinses using sterile distilled water, the seeds were left to germinate individually in glass pyrex tubes (24 x 200 mm) which contained 20 ml of water gelled with 0.6% agar (Difco) and stopped with a carded cotton. After 30 d of growth, the seedlings reached a height of approximately 70 mm with two to three epicoltyledonary nodes and an apical bud (Fig.1). The microcutting of this stock consisted of a leafed uninodal fragment of 10 to 15 mm long.

(ii) Four-year-old plants which were raised in a greenhouse and maintained in a juvenile state by regular pruning (Fig. 3). The shoot segments were taken from the non-lignified part of young, growing coppice shoots. All the leaves were removed, then the shoots were dipped for 7 min and agitated in mercurous chloride solution (1‰ $HgCl_2$ in 70% ethanol) to which was added a few drops of Tween 20. The segments were then plunged in ethanol at 70% for 30 s before being rinsed three times in sterilized, distilled water. The shoot segments were then cut in uninodal microcuttings of 10 to 15 mm in length and aseptically introduced into tubes containing the test media.

These two types of explants coming from sterile juvenile plants or 4-year-old plants cultivated in a greenhouse are referred to as "first generation cuttings". The explants obtained from the shoots produced *in vitro* from these first generation cuttings were used for the study of rooting. They are referred to as "second generation microcuttings".

Culture Methods.

*Media and Growing Conditions.*Two mineral basal media were used, according to the research objective.

(i) Elongation of shoots. For the elongation of shoots from first generation cuttings, a modified Murashige and Skoog (1962) (MS mod.) medium was used as the basal medium. The modification consisted of decreasing by half the propor- tion of macroelements. Added to this basal medium was either 6-benzyl-amino-purine (BAP) or 6-(4-hydroxy-3-methylbut-trans-2-enyl)-aminopurine (zeatin) at three different concentrations (see Results). The control medium did not contain growth regulators.

(ii) Rooting. The study of rooting was conducted on second generation microcutting generating uninodal explants, cut in the elongation phase from shoots of first generation cuttings which grew in the medium "MS mod. + zeatin $5.0x10^{-7}$ M". The rooting has an induction phase (rhizogenesis), followed by an extension phase. Two induction media were tested, for a duration of 6 d: (a) NAA $1.0x10^{-5}$ M in acqueous solution, (b) NAA $1.0x10^{-5}$ M in modified Jordan *et al.* (1978) medium (JN mod.) by decreasing by half the macroelements. Two induction durations were also tested: 6 d and 12 d. The influence of these induction factors on root extension was determined after a passage in the rooting extension medium, made from JN mod. without growth substances.

The media used for shoot multiplication and rhizogenesis contained 20 $g.l^{-1}$ of saccharose, was solidified with 6 g. l^{-1} of agar Difco and sterilized at 120° C for 15 min. The pH was fixed at 5.8 before autoclaving. The glass pyrex tubes (24 x 200 mm), plugged with non-absorbant cotton and each containing an explant, were placed in a growth chamber where the temperature was 30 ± 2° C, the humidity varied between 20 and 40% RH with a photoperiodic regime of 16 h daylight and 8 h darkness. The artificial light was furnished by Sylvania Grolux F 40 W fluorescent tubes of 12.5 $W.m^{-2}$.

Separation and acclimatization. After rooting, the micro- plants were carefully extracted from the growing tubes and were directly transplanted in pots on a substratum consisting of vermiculite mixed equally with fertile topsoil (Support NF U 44551, Ets Puteaux S.A. 78150 LE CHESNAY, France), made from peat from *Sphagnum* and *Carex*. They were thus kept for 4 d in a greenhouse under a double enclosure provided by a mini-greenhouse and a canopy of transparent plastic material (Fig. 6) at a mean temperature of 35° C, the hygrometry varying between 70 and 100% RH. During the following 7 d, the plants (having been rid of their canopies) were kept in the mini-greenhouse and were vaporized (3 times for 6 min every 24 h). The plants were then taken from the greenhouse and were misted at a temperature of 35 ± 8° C under the same watering frequency (Fig. 7).

Results and Discussion

Juvenile Stock

Shoot production and elongation. Table 1 illustrates the effect of different concentrations of zeatin and BAP on the shoot development of the cutting explants. After 60 d of

Table 1. Effect of different concentrations of zeatin and BAP on shoot development on first generation cuttings from plantlets issuing from *in vitro Acacia senegal* seedlings after 60 d. There were 24 replicates per treatment.

Zeatin (M)	BAP (M)	Survival percentage	Explants exhibiting a basal callus (%)*	Volume of basal callus (mm^3)	Mean height of shoot formed from bud of cutting (mm)	Number of nodes on new shoot	Explants having a developed node larger than 10 mm (%)*	Multiplication rate **
0	0	100	0	0	9.7 ± 3.6	2.1	46	1.4 ± 0.4
$5x10^{-7}$	0	100	92	566	35.6 ± 12.8	4.2	79	3.0 ± 0.8
$1x10^{-6}$	0	100	100	116	18.9 ± 8.0	3.0	58	2.2 ± 0.8
$5x10^{-6}$	0	100	100	263	15.5 ± 5.9	3.2	50	1.4 ± 0.7
0	$5x10^{-7}$	100	0	0	12.0 ± 6.2	2.0	46	1.9 ± 0.8
0	$1x10^{-6}$	100	100	33	11.7 ± 2.4	2.7	58	1.2 ± 0.2
0	$5x10^{-6}$	100	100	103	15.5 ± 7.9	2.8	62	1.5 ± 0.6

* As a percent of the original number of explants (24) per treatment.

** Multiplication rate = Number of uninodal explants suitable for cutting (greater than or equal to 10 mm) per primary microcutting which had developed a bud equal to or greater than 10 mm, after 60 d.

Values are mean ± 95% confidence limits.

growth, the treatment "zeatin $5.0x10^{-7}$ M" induced better shoot development than all of the other treatments, and resulted in the formation of a shoot which averaged 35.6 mm in length; the shoot had four nodes and produced three explants suitable for cuttings. A single axillary shoot formation was most often observed, in contrast to the type of multiple shoot proliferation frequently described in the literature in response to cytokinins (Al Kai *et al.*, 1984; Barghchi, 1987; Mittal *et al.*, 1989). Most of the explants (79%) which underwent this treatment developed a shoot more than 10 mm in length. When higher concentrations of zeatin were used ($1.0x10^{-6}$ M or $5.0x10^{-6}$ M), this percentage decreased markedly, as well as the number of new explants suitable for cuttings. However, the number of nodes remained approximately the same. Thus, internode length decreased. At the base of the segments, calli developed which had a greater mean volume (approx. 566 mm^3) when using a concentration of $5.0x10^{-7}$ M of zeatin than with other concentrations. The proliferation of basal calli did not seem to affect the development of the axillary shoot. No calli were observed on the control, which overall registered inferior results for all of the examined parameters.

For the BAP treatments, the elongation of the shoot from first generation cuttings was much less than that obtained with zeatin as, after 60 d, shoots reached a mean length of 11.7 to 15.5 mm as compared to 35.6 mm. After this length of time, for all concentrations of BAP tested, two to three nodes were formed on the shoot. The percentage of explants having a shoot greater than 10 mm in length was only 62%, compared to 79% for zeatin. No basal calli were observed for the lowest concentration of BAP ($5.0x10^{-7}$ M) nor for the control.

This experiment demonstrated that cytokinins were indispensable for the sprouting of the axillary buds of *Acacia senegal*, the multiplication of nodes and shoot growth. Among the cytokinins, BAP is the one which is used most frequently on the majority of woody species (Bonga, 1987) such as *Acacia koa* (Skolmen and Mapes, 1976), *Sequoia sempervirens* (Boulay, 1980) or *Acacia albida* (Duhoux and Davies, 1985); but in our experiment, BAP did not give the best results. Zeatin, a natural cytokinin, produced a better stimulation of the growth of microcuttings, confirming the works of Cañas (1988) on *Olea europea* and Elliot (1971) on apple trees.

Rhizogenesis and elongation. Table 2 shows that the mineral medium influenced the development of the plant during the root induction phase. A 6 d induction with NAA ($5.0x10^{-5}$ M) in the JN mod. medium improved all the measured parameters compared to the water + NAA medium: in 30 d the percentage of rooted plants reached 100% (Fig. 2); the number of roots increased twice. By separating the rhizogenic processes into different phases, other authors have obtained similar observations. This is the case for Depommier (1981) for *Eucalyptus sp.*, Amerson and Mott (1982) for *Pinus monticola* and Basbaa (1991) for *Gleditsia triacanthos*.

Table 2. Effect of the mineral medium on root development (30 d) and on shoot elongation (60 d) after transplanting in medium without NAA. The length of induction is 6 d. There were 24 replicates per treatment.

Induction medium	Length of time before appearance of first root (d)	Rooted plants (%)	Number of roots per rooted explant	Size of new shoot (mm)
JN mod.+ NAA*	5	100	3.5 ± 0.8	34.5 ± 5.6
Water + NAA *	0	60	1.7 ± 0.2	10.0 ± 3.3

* NAA was used at a concentration of $5.0x10^{-5}$ M.
Values are mean ± 95% confidence limits.

Young green shoots from 4-year-old stock.

Shoot production and elongation. On the MS mod. growth medium, a single branchlet was formed from the axillary bud; it grew to produce several nodes suitable for cuttings (Fig. 4). This mode of shoot production also occurs in *Leucaena leucocephala* on an optimal multiplication medium (Dhawan and Bhojwani, 1985). With respect to the control group, the length of the twig formed when zeatin was present increased by 84% at a concentration of $1.0x10^{-7}$ M, by 168% at a concentration of $5.0x10^{-7}$ M, and only 35% for $1.0x10^{-6}$ M (Table 3). In contrast, for BAP, the only significant increase (+58%) was obtained using the highest concentration ($1.0x10^{-6}$ M).

If one considers the percentage of explants having, after 60 d, developed an axillary branchlet from which a cutting can be taken, the same relationship appeared: the highest concentrations of BAP and lowest concentrations of zeatin provided the best results (Table 3). The branchlets whose length was greater than or equal to 10 mm each produced one or several segments from which cuttings can be made. The best multiplication rate was 3.3; it was obtained with zeatin at a concentration of $5.0x10^{-7}$ M. Similar results have been shown with *Prosopis cineraria* (Goyal and Arya, 1984) and *Pterocarpus santalinus* (Patri *et al.*, 1988) on their optimal multiplication medium. Zeatin produced, at the concentra- tions tested, multiplication rates superior to those obtained with BAP, whose best registered result was 2.2 at a concentration of $1.0x10^{-6}$ M.

Rhizogenesis and elongation. All of the explants undergoing a rhizogenic induction treatment in a JN mod. medium which had a high concentration of auxin ($5.0x10^{-5}$ M) produced roots. Good development of the axillary branchlet after 60 d was observed (Fig. 5). Under the experimental conditions cited herein, it is clearly evident that it is possible to obtain second generation microcuttings which are both rooted and have developed a vigorous branchlet (Fig. 5) producing, on average, 3-4 microcuttings.

Table 3. Effect after 60 d growth *in vitro* in MS mod. medium of different concentrations of zeatin and BAP on the development of cuttings taken from *Acacia senegal* coppiced plant stock 4 years of age. There were 24 replicates per treatment.

Zeatin (M)	BAP	Survival rate (%)	Length of new shoot sprouting from bud (mm)	Explants having developed a shoot equal to or greater than 10 mm * (%)	Multiplication rate**
0	0	75	10.9 ± 4.2	39	1.1 ± 0.3
$1x10^{-7}$	0	71	20.1 ± 9.2	47	1.9 ± 0.8
$5x10^{-7}$	0	71	29.2 ± 4.8	41	3.3 ± 1.0
$1x10^{-6}$	0	92	14.7 ± 8.5	27	2.3 ± 0.5
0	$1x10^{-7}$	79	11.9 ± 8.3	32	1.5 ± 1.3
0	$5x10^{-7}$	50	10.2 ± 5.8	42	1.4 ± 0.6
0	$1x10^{-6}$	67	17.2 ± 7.8	50	2.1 ± 0.5

* Relative to the number surviving.
** Multiplication rate = As detailed in Table 1.
Values are mean ± 95% confidence limits.

Conclusions

In the case of *Acacia senegal,* BAP was seen to be less effective than zeatin for the sprouting of the axillary buds and for stimulating the growth of new shoots. Zeatin, used at a low concentration ($5.0x10^{-7}$ M), allowed for a better elongation of shoots.

With *Acacia senegal* under the present experimental condition, from the axillary bud one single shoot is able to lengthen and produce several nodes suitable for cuttings, in contrast to other species which show a proliferation of stems. This method of propagation permitted a mean multiplication coefficient between 3 and 4, from explants coming from both plantlets as well as young coppice from 4-year-old stock.

By exposing the *Acacia senegal* microcuttings to a short inductive treatment with a high concentration of auxin (NAA), 100% rooting was obtained. Transferring the shoots to a medium without phytohormones permitted a vigorous development of roots. During the root elongation phase, it was observed that the rooting quality had a considerable influence on the elongation of the shoots. In effect, the shoots of the well rooted explants showed better development than those obtained from weakly rooted explants or from those which were not at all rooted. The elongation of shoots which occurred at the same time as the rooting, during this stage where phytohormones were absent, provided the advantage of continuing with the *in vitro* multiplication of *Acacia senegal*, as was the case for *Gleditsia triacanthos* (Basbaa, 1991). The acclimatization of rooted plants which were transplanted without difficulty in a greenhouse (Figs. 6, 7), indicated the reliability of the *in vitro* plant multiplication technique with regard to the species studied. The results obtained with juvenile and young green shoots from 4-year-old stock demonstrates that the large scale propagation of *Acacia senegal*, a species having multiple uses among them the production of gum arabic, can be undertaken using this method.

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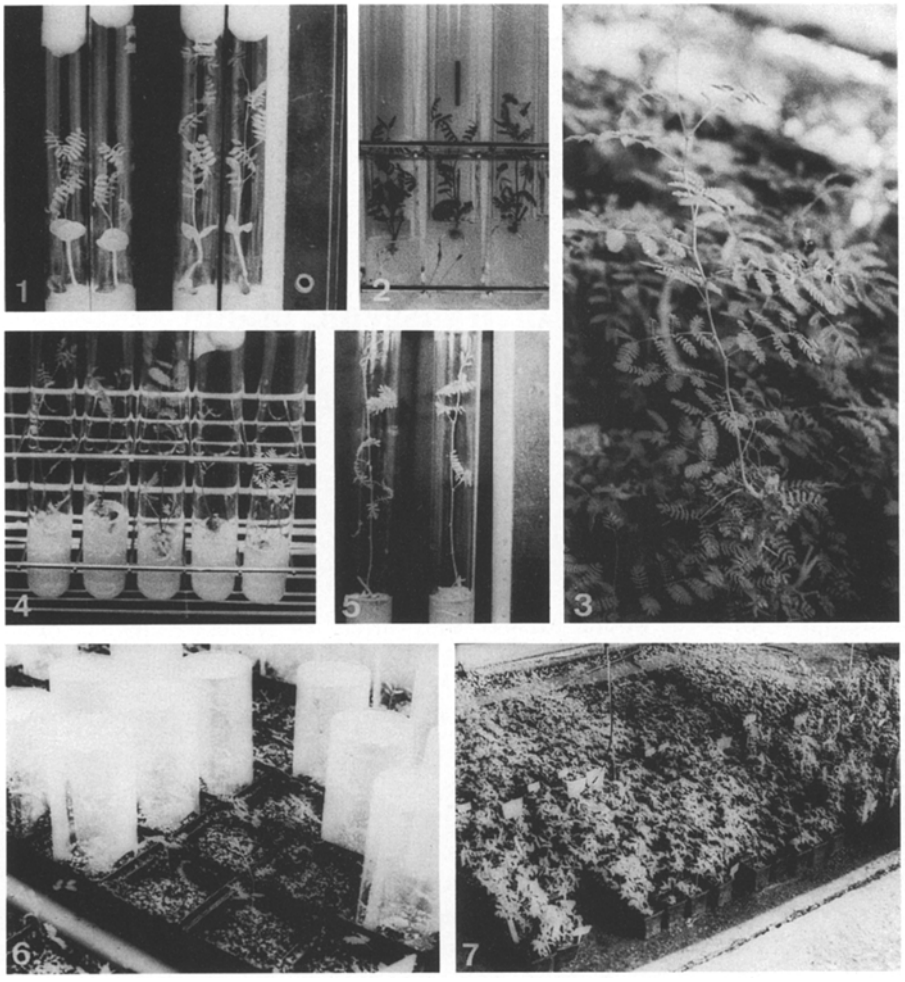

Microcuttings of *Acacia senegal* from plantlets or from young shoots taken from subjects 4 years of age.

Fig. 1. 30 d old seedlings grown *in vitro* in agar water. **Fig. 2.** Rooting of microcuttings taken from seedlings in modified Jordan's mineral medium (see text) after 30 d of growth. Roots developed from a callus. The bar represents 2 cm. **Fig. 3.** 20 d old herbaceous root formed after coppicing from 4 year old plant stock grown in a greenhouse. **Fig. 4.** Shoot extension of 60 d old microcuttings grown on the multiplication medium (MS modified and zeatin at a concentration of 5.0×10^{-7} M), issuing from 4 year old plant stock. **Fig. 5.** 60 d old second generation microcuttings in a rooting and multiplication medium (Jordan modified) after a brief time in the root induction medium (Jordan modified and NAA at 5.0×10^{-5} M). The microcuttings came from shoots produced in a multiplication medium (Fig. 4). **Fig. 6.** Plantlets of *A. senegal* after transfer to small pots containing vermiculite mixed equally with fertile topsoil. They were kept under a double enclosure provided by a mini-greenhouse and a canopy of transparent plastic material. **Fig. 7.** Acclimatized plants.

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